THE ACCUMULATION AND FATE OF BROWN PIGMENTS IN LEAVES FROM A LITTER OF *FAGUS SYLVATICA* L.: A MORPHOLOGICAL AND CHEMICAL STUDY

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ABSTRACT

Freshly deposited beech leaves were successively sampled in November and May from the litter of a brown mesotrophic soil. The dry and ground leaves were then extracted with water (WE) and a sequence of organic solvents. Transmission electron microscopy of cross-sections of leaf cuticle and parenchyma showed that opaque material accumulates as granules-and globules in the vacuoles, or forms coatings on the inner cell walls. These deposits were slightly modified after extraction with water, but were solubilized, partly in methanol (ME), mostly in dimethylformamide ($DMFA_1$ and $DMFA_2$).

The brown soluble pigments (from the water and DMFA extracts) represented 31% and 36% of the total carbon of the leaves collected in November and in May, respectively, and incorporated from 55 to 75% of the leaf nitrogen, mostly as proteinaceous material combined to polyphenolic polycondensates.

Litter decay resulted in a decrease in water solubility of the pigments, whereas the condensation degree of these pigments, as well as the resistance of their nitrogen against acid hydrolysis, increased from the November leaves to the May leaves.

INTRODUCTION

Dark-colored polymers formed during the autolysis of leaves or in soil litters represent a source of humic material, which may be as important as lignin (Kononova and Aleksandrova 1973; Metche et al. 1970; Minderman 1979). Toutain (1981) has shown that such compounds accumulate in the leaf of the beech (*Fagus sylvatica* L.), either as fine coatings and in-

crustations on the inner cell walls, or as spheroids in the vacuoles of the epiderm and parenchyma cells. Although the chemical nature of these compounds has not been clearly established, it is probable that their formation is related to the accumulation of tannic material and to the oxidation of polyphenolic monomers (Davies 1971; Haider et al. 1965). The concentration of these monomers in leaves decreases at the end of the vegetative cycle (Tissut 1968), but substantial proportions of some of them are still water-soluble (Whitehead et al. 1983). Reactions with proteinaceous compounds during oxidation yield nitrogen- containing polymers, which have been shown, in different plant materials, to present analytical similarities with phytomelanins and humic acids (Andreux 1981; Metche et al. 1970; Nicolaus 1968; Rafidison 1982).

In order to improve our knowledge regarding the abundance and nature of these brown pigments in the litter of beech leaves, their solubility in water and in a sequence of organic solvents was determined. The effect of each reagent was controlled by electron microscopy, and the amounts extracted from freshly deposited leaves were compared with those obtained from partly decayed material.

MATERIALS AND METHODS

Leave Material

The upper 0-2 cm layer of the litter of a brown mesotrophic soil was collected in November (N leaves) and in May (M leaves), air-dried and ground, either to 80 μm for analytical purpose, or to 4 mm, for the ultrastructural observation.

Extraction Procedure

Ground material (200 g) was dispersed in 2 l of distilled water containing 5% of chloroform, allowed to stand at 4°C, with periodical stirring, for one week, then filtered on glass fiber filter, and finally rinsed with water. An aliquot portion of the filtrate (WE) was diluted in acetone (2:1 v/v) and the mixture was allowed to stand overnight at room temperature. The brown precipitate which appeared after this treatment (WEP) was then centrifuged at 6000 g.

The water-extracted leave material was fractionated with a procedure similar to that described by Thompson et al. (1972), using exhaustive extraction with organic solvents. Cold extractions carried out with methanol, chloroform, ethyl acetate and aceton + water (1:1 v/v) yielded the following fractions: ME, CH, EA and AW, respectively. Two extractions under

reflux at 120°C were then performed with dimethylformamide, and the corresponding $DMFA_1$ and $DMFA_2$ fractions were separated. The residue, which had a grey colour, was rinsed with diethyloxide and dried.

Chemical Analysis

The water and organic extracts were dried at 40°C under low pressure, weighed, and their carbon and nitrogen contents were determined with a "CARLO ERBA" 1106 analyzer. The WE, WEP and DMFA fractions were hydrolysed with 3 N HCl under reflux for 16 hours. After cooling the reaction products, the solid residue was separated by centrifugation at 6000 g, dried and weighed. On the supernatant, total carbon and nitrogen were determined, and the concentrations of ammonium and amino-acids were measured respectively by steam-distillation at pH 9.2 (Stevenson et al. 1967), and by colorimetry in the presence of ninhydrine reagent.

Ultrastructural Studies

After each extraction of the leaves ground to 4 mm, an aliquot portion of the residue was collected, and dried under vacuum. These dry materials were successively dipped for one hour in a 2.5% (v/v) solution of glutaraldehyde in pH 7.2 phosphate buffer, and in a solution (1:1 v/v) of osmium tetraoxide in the same buffer. After washing with the buffer alone, the samples were then dehydrated with acetone, prior to inclusion in an "EPON 812" resin. Thin sections were then prepared with a OMV2 REICHERT microtom, stained with uranyl acetate and lead citrate (Reynolds, 1963), and observed with a transmission "ZEISS EH 92-2" electron microscope.

RESULTS

Distribution of Extractable Materials (Fig. 1, Table 1).

The November leaves contained more than 17% (w/w) of water-soluble (WE) material (Fig. 1), the elemental composition of which was close to that of the whole leaves (Table 1). The acetone-precipitated WEP fraction represented about 5% (w/w) of the dry leaves. Methanol extracted a similar amount of material as water did, but no nitrogen was found in the extracts. Chloroform and ethyl acetate + water extracted not more than 5% (w/w) of the leaf carbon and nitrogen. DMFA extracted dark material, which represented 13.5% (w/w) and 54% (w/w) of the leaf carbon and nitrogen, respectively. The C/N ratios of $DMFA_2$ was lower than that of $DMFA_1$, but both C/N ratios were much lower than those of the whole leaves and other extracts (Table 1).

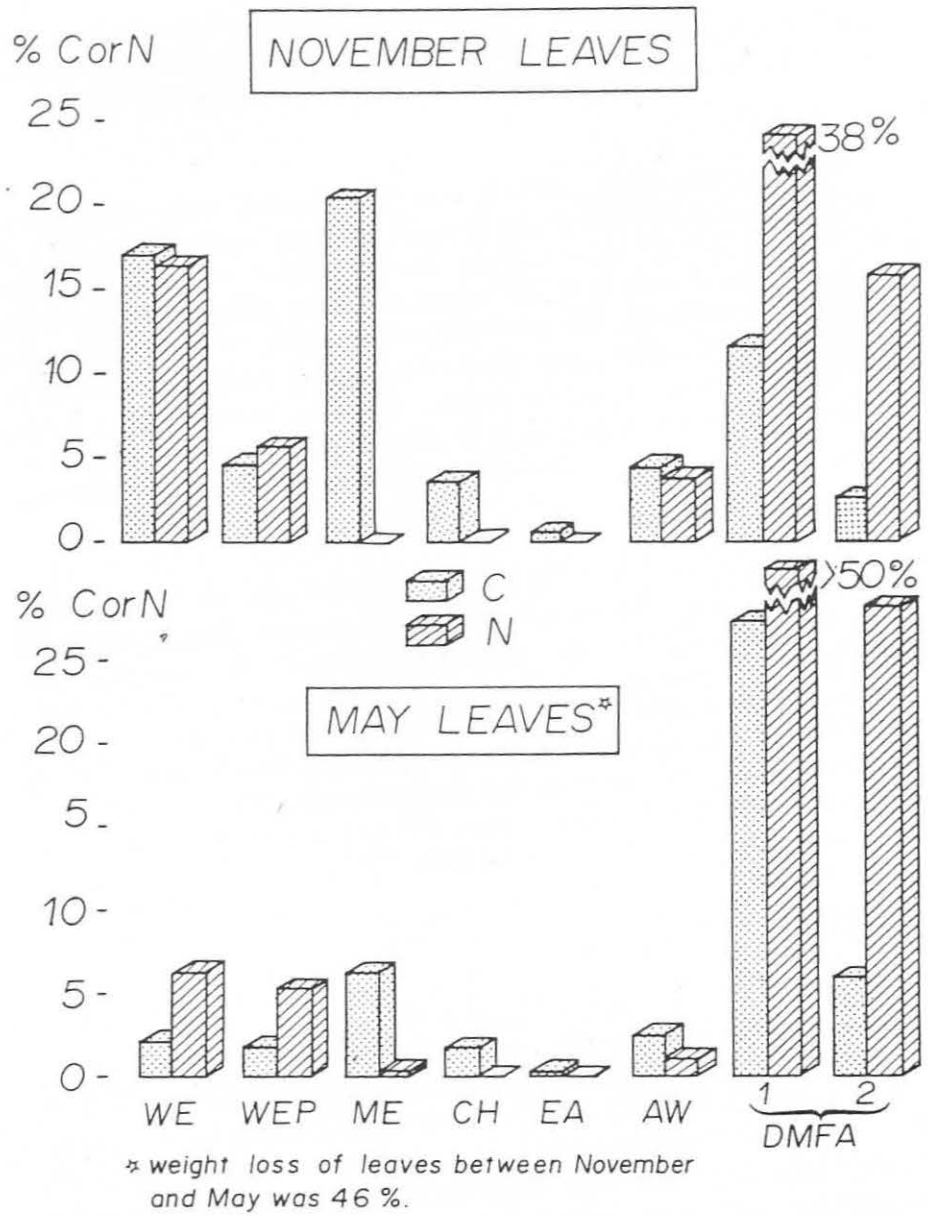

FIGURE 1. Solubility, in water and organic solvents, of carbon and nitrogen from leaves sampled in November and May.

The May leaves contained only 2.8% (w/w) of water-soluble (WE) material. This material was almost completely precipitated with acetone, and had a lower C/N ratio (18) than the whole leaves. Methanol, ethyl acetate and acetone + water extracted lower amounts of material than from the N leaves, whereas DMFA extracted 28.9% (w/w) of leaf carbon and about 75% of leaf nitrogen.

TABLE 1. ELEMENTAL ORGANIC ANALYSIS OF LEAVES AND LEAF EXTRACTS.

Material	November leaves			May leaves		
	C %	N %	C/N	C %	N %	C/N
Whole leaves	47.6	0.72	66	45.9	0.94	49
WE[a]	46.2	0.67	69	37.8	2.14	18
WEP	39.4	0.74	53	37.1	2.20	17
ME	66.2	tr[b]	nd[c]	61.1	0.14	436
CH	83.9	tr	nd	73.0	tr	nd
EA	70.9	tr	nd	61.2	tr	nd
AW	55.1	0.73	75	49.5	0.47	105
$DMFA_1$	52.3	2.53	20	53.4	2.95	18
$DMFA_2$	47.8	4.23	11	49.3	4.71	10

[a] The meaning of symbols are reported in the Materials and Methods.
[b] Traces.
[c] Not determined.

Composition of Brown Pigments Extracted With Water and DMFA (Table 2).

The acetone precipitates WEP from the water-soluble fraction, and the $DMFA_1$ extracts were the only fractions which had a brown color, and contained a proportion of N equal to, or higher than that of the whole leaves. Although the WEP fraction from the November leaves contained three times less organic nitrogen than the WEP fraction from the May leaves, both fractions contained similar amounts of carbon and hydrogen: H/C atomic ratios reached values of 1.6 to 1.7, which corresponded to a predominant proportion of aliphatic material.

Acid hydrolysis of the WEP fraction from the November leaves dissolved more than 50% of this material, and 100% of its nitrogen compounds. About 46% and 21% of this nitrogen were as amino-acids and ammonia respectively, but 33% were not identified. Because of the lack of material, no further analysis was done of the WEP fraction from the May leaves.

No significant difference was observed on the composition of the DMFA fractions from the November and May leaves. These fractions contained

less oxygen than the WEP fraction from the November leaves and an equal amount of nitrogen as the WEP fraction from the May leaves. Their H/C ratios were lower than that of the WEP fractions.

Acid hydrolysis dissolved 49.1% (w/w) of the $DMFA_1$ fractions from the November leaves, but only 20% of that from the May leaves. Larger proportions of nitrogen were acid-soluble, but the proportion of unhydrolysable nitrogen was only 8% in the November leaves, whereas it was 22% in the May leaves. Amino-acids represented only 40% of hydrolysable nitrogen in the $DMFA_1$ fraction from the N leaves, but almost 70% in the $DMFA_1$ from the M leaves. Ammonia did not exceed 5%, and the rest was unidentified.

TABLE 2. COMPOSITION OF ACID HYDROLYSATES OF PURIFIED BROWN PIGMENTS EXTRACTED WITH WATER AND DIMETHYLFORMAMIDE FROM NOVEMBER AND MAY LEAVES.

Material	Hydrolyzable material % of total			Forms of nitrogen % of total N		
	Weight	C	N	NH_4	α-Amino N	Unknown N
November leaves						
WEP	68	57	100	21	46	33
$DMFA_1$	43	44	92	4	38	50
May leaves						
WEP	(No material)					
$DMFA_1$	nd[a]	21	75	5	54	19

[a] Not determined.

Ultrastructural Study of the Effects of the Extractants (Fig. 2).

The present study was limited to the freshly deposited N leaves. As previously shown by Toutain (1981), the epiderm cells of these leaves contain a variable density of dark, fine granules and coatings, which appear more opaque than the cell walls on which they seem to adhere (Fig. 2.a). In the parenchyma, opaque material concentrates as large spheroids and irregular granules in the vacuoles, and as thick coatings of the inner cell walls.

The opaque granules, spheroids and coatings which were observed on the dry untreated leaves were not significantly modified after extraction with water (Fig. 2.b). Methanol and chloroform neither affected the general aspect of the material, except in some cases, in which a lower density of granules was observed (Fig. 2.c). Treatment with acetone and water dis-

solved most of the spheroids, and brought about a homogeneous dispersion of the granules on the majority of the cells (Fig. 2.d).

Extraction with DMFA seemed to wash out these opaque granules, as most of the cells appeared empty after this treatment (Fig. 2.e).

The aspect of the cell walls was also slightly modified, probably as a consequence of heat treatment: the plasmic membranes in the epiderm cells separated from the external cell wall, and the opacity of the pecto-cellulosic laminae between the parenchyma cells decreased (Fig. 2.f). However, no evidence of etching of the lignin thickenings was observed.

DISCUSSION AND CONCLUSIONS

More than 40% of the total organic carbon of the brown leaves of the beech were shown to be soluble in water and a sequence of organic solvents. In freshly deposited leaves, which were sampled in November, about one third of this soluble fraction was extracted with cold water, mostly as small molecules, which disappear rapidly during further decay and leaching of the leaves. Only a small proportion of the water-soluble fraction was formed by brown, nitrogen containing, polymeric compounds, which remained extractable after the period of Winter decay.

Cold organic solvents eliminated the lipophilic leave pigments, but were generally inefficient in dissolving the brown material. Only methanol yielded a significant amount of reddish material, but no nitrogen was extracted in any case. DMFA extracted the majority of the brown material, in similar proportions as with 0.1 N NaOH, as shown by Rafidison (1982), who concluded that there existed an analogy between this material and soil humic substances. As a result, the grey powder obtained after washing and drying the leave residues was mostly formed by cellulose and lignin, although a slight proportion of unextracted pigment was probably present.

Comparison of leaves sampled in November and May indicated that the materials soluble in water and in cold solvents decreased during Winter decay. Selmi (1975) and Bartoli and Selmi (1977) have shown that the corresponding weight loss was 46%, as a consequence of leaching of water-soluble material, and partial degradation of lignin and cellulose. Contrarily, the relative concentrations of DMFA-soluble pigments increased in the meantime from 13.5% to 28.9% on the basis of the final weight, and to 18.6% on the basis of the initial weight. No significant difference with season was found in the elemental analysis of the DMFA-soluble pigments, in which more than 60% of the total leave nitrogen were incorporated.

The high nitrogen content of these pigments is probably the result of the stabilization of protein nitrogen through reaction with phenolic poly-

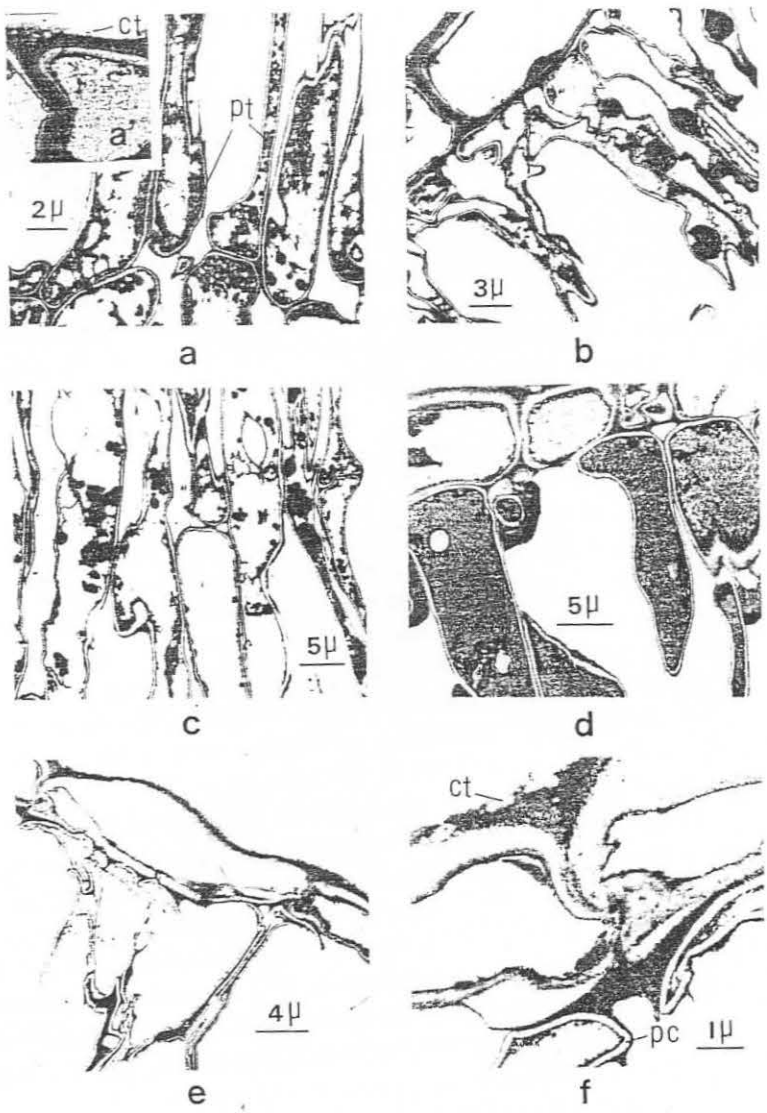

FIGURE 2. Transmission electron microscopy of brown November leaves before and after extraction with solvents.

a, a'. Untreated leaves: general aspect (a) and detail (a') of epiderm and parenchyma cells.

b. Parenchyma cells after extraction with water.

c. Parenchyma cells after extraction with methanol and chloroform.

d. Parenchyma cells after extraction with aceton + water.

e. Epiderm cells after extraction with DMFA.

f. Aspect of the parenchyma cell-wall after extraction with DMFA.

Symbols in the pictures indicate the cuticle (C_t), pigment spheroids or granules (pt) and pectocellulosic laminae (pc).

mers (Andreux 1981; Davies 1971; Haider et al. 1965; Minderman 1979). The stabilization and polycondensation seemed to increase with increasing decay, as suggested by the resistance of nitrogen to hydrolysis, and the decreasing proportions of proteinaceous nitrogen, from November to May. Similar observations were done on whole leave hydrolysates by Janel et al. (1979), who pointed out the major role of polycondensation reactions in the stabilization of nitrogen.

Some structural properties of these pigments were also suggested by their morphological study. The opacity of the brown pigments to the electrons resulted partly from their polymeric structure, but was considerably increased by previous staining.

This reactivity was probably the result of the abundance of surface carboxylic charges, as shown by infra-red spectroscopy and base titration (Rafidison 1982). Whereas other leave components soluble in water and cold solvents were almost totally transparent, the DMFA-soluble pigments were much more opaque than the cell-wall constituents. The globular aspect of part of this material may be related to a hydrophilic behaviour, as a consequence of the leaf senescence and drying. Such a behaviour was suggested by the superficial destruction of the globules after treatment with lipophilic solvents.

No definite conclusion could be drawn regarding the possible degradation of the cell structure by the action of DMFA and heating: no superficial etching of the lignin thickenings was observed, but the aspect of the pectin laminae was slightly modified. However, only 2.2% (w/w) of sugars were found in the purified pigments, whereas phenolic monomers related to lignin were insignificant in their sodium-amalgam degradation products (Rafidison, 1982).

In conclusion, this study shows that extraction with DMFA can be proposed, in place of alkaline water reagents, to selectively isolate a maximal proportion of brown pigment from decaying leaves, with minimal dissolution of structural cell material. These pigments, which may incorporate the whole of the leaf proteinaceous material, probably represent the primordial form of humic polycondensates in beech forest soils.

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